HHS Public Access
Author manuscript
J Med Chem. Author manuscript; available in PMC 2019 September 27.

Published in final edited form as:
J Med Chem. 2018 September 27; 61(18): 8104–8119. doi:10.1021/acs.jmedchem.8b00589.

Advances and Insights for Small Molecule Inhibition of Macrophage Migration Inhibitory Factor

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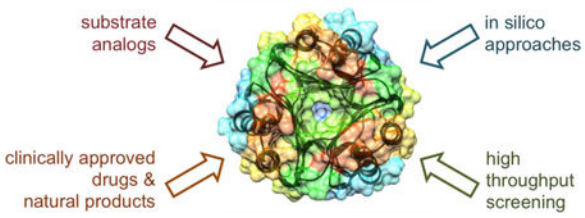
Abstract

Macrophage migration inhibitory factor (MIF) is an upstream regulator of the immune response whose dysregulation is tied to a broad spectrum of inflammatory and proliferative disorders. As its complex signaling pathways and pleiotropic nature have been elucidated, it has become an attractive target for drug discovery. Remarkably, MIF is both a cytokine and an enzyme that functions as a keto-enol tautomerase. Strategies including *in silico* modeling, virtual screening, high-throughput screening, and screening of anti-inflammatory natural products have led to a large and diverse catalog of MIF inhibitors as well as some understanding of the structure–activity relationships for compounds binding MIF's tautomerase active site. With possible clinical trials of some MIF inhibitors on the horizon, it is an opportune time to review the literature to seek trends, address inconsistencies, and identify promising new avenues of research.

Graphical Abstract



1. INTRODUCTION AND BIOLOGY

Macrophage migration inhibitory factor (MIF) is a pleiotropic protein implicated in the pathogenesis of many infectious and autoimmune diseases. Although originally identified in the 1960s as a soluble factor capable of eliciting the behavior for which it is named, cloning and expression of the protein was not accomplished until 1989.[1–3] Subsequently, MIF was rediscovered as a cytokine,[4] enzyme,[5] hormone,[6] chemokine,[7] and molecular chaperone.[8]

The MIF protein is a 115-amino acid polypeptide that folds to form two antiparallel α-helices that pack against a four-stranded β-sheet. The oligomerization state of MIF has been a subject of speculation, with various reports claiming the protein to exist as a monomer,[9]

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Notes
The authors have filed patent applications and have issued patents for MIF inhibitors.

dimer,[10] or trimer.[11] The general consensus, however, is that MIF's de facto structure is a homotrimer which is toroidal in shape with a central, solvent-filled pore. MIF crystallizes as the trimer,[12,13] and studies aiming to resolve the enigma of MIF oligomerization have confirmed the prominence of the trimer in solution and its recognition by MIF receptors.[14,15] However, biological roles of MIF monomer remain possible: incompetent MIF monomer is known to exist in solution,[14] a 16-residue MIF peptide fragment has been shown to reproduce some of the biological activity of the full protein,[16] and studies have suggested the chaperone functions of MIF might be driven by the monomer.[8] It is entirely possible, then, that the oligomerization state of MIF is more dynamic than previously expected, with different roles existing for monomeric and trimeric protein.

The MIF trimer shares its architectural structure (but less than 20% sequence homology) with two bacterial isomerases, 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI) and 4-oxalocrotonate tautomerase (4-OT), which affect their activity through a catalytic N-terminal proline.[17] MIF contains an initiating methionine that is removed during protein maturation, resulting in a proline at the N-terminus.[18] MIF numbering in the literature can vary depending on whether or not this methionine is counted; numbering in this Perspective assigns proline as the first residue. Consistent with its structural similarity to CHMI and 4-OT, MIF was found to catalyze the keto-enol tautomerization of D-dopachrome[5] and 4-hydroxyphenylpyruvate (HPP).[19] MIF's active site is a cylindrical cavity, located at the interface of monomer subunits in the trimer.[20] Due to a local hydrophobic environment, Pro-1 has a pK_a of 5.6, around 4 pH units lower than the pK_a of proline amide, and is the most nucleophilic residue in the folded protein.[21] Despite evolutionary conservation of tautomerase activity, natural substrates of MIF have remained elusive. The D-isomer of dopachrome is non-physiological, and the high K_m of HPP and its separate localization from MIF in the body make it an unlikely substrate.[19] A neuroprotective role for MIF's enzymatic activity has been proposed following the discovery that it can catalyze the conversion of a series of toxic oxidized catecholamines to 5,6-dihydroxyindole derivatives that may serve as percursors to neuromelanin.[22] However, these findings remain to be validated in a biological system. Indeed, as of this writing, a purpose for MIF's tautomerase activity has yet to be agreed upon, and this function is largely believed to be vestigial in humans.[23] Biochemical and mutational studies have provided credibility to this theory, with multiple catalytically inactive MIF homologues retaining their cellular functions.[24–26]

In addition to tautomerase activity, MIF has been found to possess thiol-protein oxidoreductase (TPOR) activity and is capable of catalyzing the reduction of insulin and 2-hydroxyethyldisulfide.[27] A conserved Cys-56-Ala-Leu-Cys-59 (CALC) sequence located in the central pore has been identified as the catalytic domain, with the two cysteine residues being essential for TPOR activity. While this activity has led to the suggestion that MIF may be involved in the regulation of cellular redox processes,[28] recent studies have shown that MIF is capable of interconverting between a reduced (redMIF) and a pathologically implicated oxidized (oxMIF) state with antibodies targeting the CALC motif capable of distinguishing the two forms.[29] A third cysteine (Cys-80) distal to the CALC motif has been found to operate as a switch, converting redMIF to oxMIF through posttranslational modification.[30] The structural rearrangements incurred by activation of this "switch cysteine" include a conformational change of the CALC motif that would allow Cys-56 and

Cys-59 to undergo disulfide shuffling, a function which might be essential for TPOR activity.

Though MIF's catalytic functions are indeed intriguing, it is MIF's involvement in disease-related signaling that has warranted the continued interest in this protein. Its role as a cytokine, for example, is compelling not least because of its peculiarity when compared to other proteins in this family. Unlike other cytokines, MIF exists preformed in multiple cell types throughout the body[31,32] but especially in cells of the nervous system,[33] endocrine system,[32] and in cells that have direct contact with the natural environment (e.g., lung, skin, and gastrointestinal cells).[31,34] Upon stimulation with lipopolysaccharide (LPS) or other activating stimuli, cells secrete preformed MIF into the extracellular milieu. Lacking an N-terminal signal sequence, MIF is released from the cell via a non-classical transport mechanism involving complexation and co-secretion with the golgi-associated protein p115.[35] Extracellular MIF is capable of upregulating the production and release of the interleukin IL-8 and is synergistic with LPS in the production of other pro-inflammatory cytokines, including tumor necrosis factor (TNF-α) and IL-1β.[36] It additionally differs from other cytokines in its ability to override the anti-inflammatory effects of glucocorticoids. Low concentrations of glucocorticoids actually induce the secretion of MIF, which counter-regulates glucocorticoid anti-inflammatory activity through the restoration of cytokine (TNF-α, IL-6, IL-1β) production.[37] It has been hypothesized that MIF and glucocorticoids in fact form a regulatory dyad of the innate immune response, simultaneously curtailing the development of life-threatening infections and runaway inflammation.[38] Reports have identified the phosphatase enzyme MKP-1 and possibly the transcription factor NF-κB along with its inhibitory counterpart IκBα as mediators of crosstalk.[39–41]

Many of MIF's effects are mediated through interactions with the receptor CD74.[42] MIF and CD74 associate into a signaling complex with CD44, which can activate the mitogen activated protein (MAP) kinase cascade, resulting in phosphorylation and sustained activation of the extracellular signal-regulated kinases ERK1/2.[43] One downstream effect of this pathway is inhibition of p53-mediated apoptosis,[44,45] which has warranted considerable interest, as it allows for both accumulation of oncogenic mutations and sustained inflammation through enhanced survival of activated macrophages, suggesting a mechanism by which MIF may enact some of its pathology. MIF additionally interacts with the chemokine receptors CXCR2, CXCR4, and CXCR7 to incur arrest and/or chemotaxis of neutrophils/monocytes, T cells, and B cells.[7,46] All three of these receptors have been shown to oligomerize with CD74, and some of these heteromeric complexes are reported to mediate certain MIF activities, including activation of the PI3K/Akt pathway, phosphorylation of JNK, and gene expression of IL-8.[47,48]

In addition to its extracellular activities, MIF is capable of undergoing endocytosis to interact with intracellular proteins. Jun-activation domain-binding protein 1 (JAB1), for example, may function in a negative feedback capacity with respect to MIF's proliferative effects. MIF–JAB1 binding arrests cell growth by attenuating JAB-1-mediated activation of the transcription factor AP-1.[49] JAB1 may also act as a molecular sink, preventing cellular release of MIF through intracellular binding.[50] More recently, MIF was found to interact intracellularly with thioredoxin-interacting protein (TXNIP), an inhibitor of NF-κB

activation.[51] MIF–TXNIP binding was shown to induce activation of NF-κB and expression of proliferative genes. MIF has also been shown to induce expression of TLR4,[52] upregulate production of matrix metalloproteinases (MMPs),[53–55] stabilize the association between p53 and its regulator Mdm2,[56] modulate the activity of insulin,[57] and act as a molecular chaperone in heat stress8 and neurodegenerative disease models.[58] Additionally, a human homologue has been identified in D-dopachrome tautomerase (D-DT or MIF-2) which is also expressed in multiple cell types, participates in similar signaling through CD74-binding, and seemingly works cooperatively with MIF to induce the production of growth and pro-inflammatory factors.[59] Indeed, the MIF interactome is extensive, and the protein's roles in cellular signaling continue to be elucidated (Figure 1).

Reflective of its expression in numerous cell types and of its diverse biological functions, MIF has been implicated in multiple infectious and autoimmune diseases, including toxic shock syndrome,[4,60] lupus,[61] rheumatoid arthritis,[62] atherosclerosis,[63] asthma,[64] colitis,[65] and diabetes.[66] It has been tied to neuroinflammation and the progression and severity of Alzheimer's disease[67] and spinal cord injury.[68] MIF's inflammatory, chemotactic, and proliferative capacities have been shown to contribute to cancer severity, and it is overexpressed in multiple models of cancer, including prostate cancer,[69] breast cancer,[70] neuroblastoma,[71] and leukemia.[72] MIF's pleiotropy has led some to suggest that it may be a key link bridging the signaling pathways associated with both inflammation and cancer.[73,74]

A genetic component of MIF pathology has been identified in a polymorphic, tetranucleotide sequence (CATT) within the *MIF* gene promoter sequence. The CATT sequence is represented between five and eight times, and progressively higher expression levels of MIF are associated with increases in the number of repeats.[75] Higher CATT repeat alleles have additionally been shown to confer increased susceptibility to diseases such as asthma,[76] cystic fibrosis,[77] and rheumatoid arthritis.[62] Altogether, MIF appears to be a crucial upstream regulator of a number of cellular processes whose dysregulation can lead to disease states. For this reason, it is a compelling target for exploration of potential therapeutics for inflammatory and proliferative disorders.

2. MIF INHIBITORS

2.1 TAUTOMERASE SUBSTRATE ANALOGS

The tautomerase activity of MIF proved an irresistible feature for early researchers of the protein and paved the way for the development of MIF inhibitors. It was originally speculated that MIF could exact some of its biological activities via an enzymatic mechanism. Thus, the tautomerase active site was seen as the logical binding site for potential inhibitors of MIF activity, and the two non-physiological substrates D-dopachrome (dopa) and HPP provided convenient scaffolds for the development of early inhibitors (Figure 2).[78–80] The discovery that the covalent inhibitor NAPQI (**1**), a primary metabolite of acetaminophen bearing structural similarity to D-dopachrome, attenuated not only tautomerase activity but also glucocorticoid overriding activity served as proof of concept that compounds binding to the enzymatic pocket have the potential to disrupt MIF recognition and cellular functions.[81] The composite inhibition of MIF tautomerase and biological activity may provide some insight into the evolutionary conservation of the active

site: if the residues in or proximal to this region are essential for receptor recognition or activation, then there would be pressure to conserve them in order to perpetuate MIF functions.

Future work capitalized on this discovery, and a number of inhibitor classes bearing key features of the dopachrome scaffold were reported and shown to attenuate diverse biological activities of MIF.[82–86] The most extensively studied of these is the isoxazole inhibitor ISO-1 (**4**), which was reported by Lubetsky et al. in 2002.[87] While it is a modest inhibitor of tautomerase activity (most reports agree on an IC_{50} value close to 18 μM),[84,88] it has been shown to inhibit many of MIF's biological functions, including glucocorticoid overriding,[87] nuclear translocation of NF-κB,[89] cellular release,[90] and cytokine-induced beta cell death.[84] ISO-1 and its derivatives have also shown anti-MIF activity in a number of *in vivo* mouse studies, including improved survival in endotoxemia,[89] colitis,[91] melanoma, and colon cancer.[92] X-ray crystallography of ISO-1 bound to MIF revealed important interactions with Lys-32, Ile-64, and Asn-97 (Figure 3). The phenolic ring of ISO-1, which forms a hydrogen bond with Asn-97 at the back of the active site, has become a common motif in deep pocket binders of MIF. Cheng et al. showed that ortho-fluorination of the phenol could improve protein–ligand affinity by strengthening this hydrogen bond through inductive effects (compound **6**), another strategy that has since been employed.[93]

2.2 MIF INHIBITORS IDENTIFIED BY *IN SILICO* METHODS

The application of *in silico* methods to drug discovery has become standard in recent decades, and for good reason: computational approaches provide a rapid, cost-efficient route to screening and filtering large libraries of compounds, visualizing protein–ligand interactions, and justifying scaffold derivitization prior to the investment of synthetic effort. MIF is no outlier in this regard with structure-based virtual screening, fragment screening, ligand-based screening, and *de novo* design being used to identify and develop novel MIF inhibitors.[94]

Availability of high-resolution X-ray crystal structures of MIF paved the way for structure-based drug design. The first MIF inhibitors discovered by virtual screening were a series of coumarin and chromen-4-ones.[95] The fourteen reported compounds lacked the structural diversity that is often the aim of virtual screening, but a very active compound was found in Orita-13 (**7**) (Figure 4). Subsequent virtual screenings have resulted in the discovery of multiple novel classes of compounds that inhibit a range of MIF's biological activities. A virtual screening conducted by our laboratory and collaborators identified 36 diverse hits from the ZINC and Maybridge libraries, two of which were pursued for further development: a series of benzoxazolones that inhibited CD74 binding and ERK1/2 phosphorylation and a series of benzisothiazoles that inhibited the same cellular functions and bound MIF either covalently or reversibly depending on substitution patterns.[96–98] A series of benzoxazol-2-thiones bearing structural similarity to the benzoxazolones has recently been reported by Le Hiress et al.[99] The derivatives were screened for activity in DU-145 cells, a prostate cancer cell line that relies on MIF for survival. The most potent compound, **10**, showed 84% inhibition of cell survival at a concentration of 100 μM.

El Turk et al. performed a virtual screening of the ChemBridge library and identified 15 inhibitors (including **11** and **12**) with tautomerase IC_{50} values ranging from 50 nM to 62 μM.[100] The most potent compound, **11**, was found to be a covalent inhibitor by mass spectrometry. Finally, a screening of both the Specs and ChemBridge libraries by Xu et al. resulted in the discovery of 10 MIF inhibitors; among them were two acylthiourea-containing compounds, including **14**.[101] Although these compounds were initially expected to inhibit MIF reversibly, subsequent work found them to be covalent inhibitors.[102] Development of this series resulted in the compound Z-590 (**15**), which inhibits a number of MIF functions, including tautomerase activity, glucocorticoid overriding, MAP kinase phosphorylation, and activated microglia-mediated neurotoxicity.[103] It was also shown to promote survival in a mouse model of sepsis.

An alternate approach to structure-based screening of libraries of drug-like molecules is the docking of smaller molecular fragments with the intention of identifying a number of weak binders that can be linked together into a potent inhibitor. This fragment-based screening approach was used by McLean et al. to identify molecules capable of binding to a cryptic site at the surface of the tautomerase active site. This site emerges by rotation of Tyr-36 to form a narrow, hydrophobic cleft with Trp-108.[104] The authors also showed through X-ray crystallography of **16** that a MIF inhibitor is capable of simultaneously occupying the canonical binding pocket and the cryptic site (Figure 5).

By 2013, diverse MIF inhibitors had been reported, and Al-Sha'er et al. used these findings to advantage and constructed quantitative structure activity relationship (QSAR) pharmacophore models, which were used in a ligand-based virtual screening of the National Cancer Institute (NCI) molecular database.[105] Nine MIF inhibitors were reported from the study, with the most potent, **17**, showing 80% inhibition of MIF tautomerase activity at a concentration of 10 μM.

A final computational approach that has been used to great effect in the discovery of MIF inhibitors is *de novo* design. Our lab has used the program BOMB,[106] which can build libraries of analogs starting from a core placed in the binding pocket, to develop a series of biaryltriazoles. The first generation of these compounds contained three analogs that showed MIF agonist activity in tautomerization, CD74 binding, and ERK1/2 phosphorylation assays.[107] However, more recent results from a tautomerase assay performed in-house have shown compound **18** to behave as an antagonist with a K_i of 37 μM. Agonist effects by these inhibitors on receptor-binding and downstream MAP kinase phosphorylation have not been retested. Progressive development of this series, including *ortho*-fluorination of the phenol and aromatic extension of the scaffold to benefit from aryl–aryl interaction, has resulted in numerous significantly more potent inhibitors, such as the quinoline **20**, which has a K_i of 57 nM (Figure 6).[108] The quinolines are the first series to undergo extensive lead optimization to yield low-nanomolar inhibitors with supporting protein crystal structures. A compound in this series has also been developed into a fluorescent probe (**22**) by tethering fluorescein to the scaffold. This probe was used to develop a fluorescence polarization assay, which presents a convenient method for determining the dissociation constant (K_d) of MIF inhibitors as an alternative to K_i or IC_{50} values obtained from tautomerization assays.[109] Good accord was found between K_d and K_i values for 20 inhibitors, giving confidence in the

accuracy of both measurements.[109] The fluorescein-tagged inhibitor may additionally prove useful for high-throughput screening and for probing the cellular activities of MIF.

Our laboratory has also used the BOMB software to help guide the development of a series of pyrazole-based inhibitors that bind inside the tautomerase active site, forming two hydrogen bonds with Asn-97.[110] The parent compound of this series, **23**, identified via virtual screening, was a relatively poor inhibitor of tautomerase activity (K_i = 113 μM), but optimization directed by *in silico* modeling and crystallography led to very potent inhibitors, including **25** (K_i = 67 nM), which is nearly 2000 times more active. The pyrazole moiety additionally functions as an isostere of the commonly employed phenol motif, and it may address metabolic concerns associated with the latter.[111]

While the efforts presented have succeeded in identifying novel MIF inhibitors, there are certain limitations of *in silico* methods that are worth mentioning. Along with inaccuracies in scoring, another shortcoming is that docking procedures are unable to predict whether an inhibitor binds MIFs tautomerase active site reversibly or irreversibly. In the past, compounds that dock to this site in ways that indicate reversible binding have later been shown experimentally to be covalent inhibitors.[102,112] The highly nucleophilic Pro-1 is the site of adduct formation. The low p*K*a of this residue should be taken into account when determining its protonation state in computational models. Compounds that dock to the active site in such a way to position an electrophilic moiety near Pro-1 have the potential to be covalent inhibitors of MIF.[101] To reduce the risk of misreporting an inhibitor's mode of action, the binding modes of compounds identified via virtual screening should be verified experimentally. Variation of incubation conditions during tautomerization assays to identify slow binders[102] and jump dilution experiments, which measure the restoration of tautomerase activity after a rapid and large dilution of the MIF–inhibitor complex,[113] are two convenient ways to determine covalent interactions. The binding mode of suspected irreversible inhibitors can then be validated via mass spectrometry analysis or X-ray crystallography.

With regard to MIF, all screening and design efforts have focused on the tautomerase active site. While this binding site is the most convenient, if not the most logical, region of the protein to target, inhibition of MIF's tautomerase activity is neither necessary[114] nor sufficient[88,115] for inhibition of its biological activities. It is worth reiterating that while inhibition of MIF's enzymatic activity is a convenient metric for determining the binding affinity of new compounds, a purpose for this function has yet to be determined. While MIF is believed to interact with its receptors CD74 and CXCR4 in the proximity of the tautomerase active site,[116–118] the MIF interactome is large, and interactions with other proteins may occur distal to this site.[49,119] The use of *in silico* methods to identify allosteric inhibitors with unique mechanisms of attenuating MIF's biological activities proves a difficult task given the limited data on MIF–receptor interactions. Nevertheless, as more becomes known of these interactions, virtual screening of alternative regions may provide a practical method for identifying allosteric inhibitors that attenuate signaling through specific binding partners. To date, other approaches have been used to introduce the necessary element of serendipity to the drug discovery process to identify such compounds.

2.3 HIGH-THROUGHPUT SCREENING

High-throughput screening (HTS) is an alternative approach to virtual screening in the detection of novel inhibitors of a protein target. Unlike the aforementioned *in silico* methods, where a protein crystal structure, homology model, or ligand model is a prerequisite, the primary requirement of a HTS approach is a scalable assay capable of efficiently screening the activity of many thousands of compounds. MIF's enzymatic activity proves invaluable in this regard, as the standard tautomerase assays used in pioneering studies could be optimized for use in HTS.

In contrast to the popularity of *in silico* methods to identify MIF inhibitors, there are comparatively few reports of HTS efforts.[113,120–122] Nevertheless, a number of novel MIF inhibitors have been identified via HTS (Figure 7). One of the most interesting of these compounds is the anti-inflammatory drug ebselen (**29**).[121] Ouertatani-Sakouhi et al. reported that ebselen covalently modifies Cys-80 and inhibits enzymatic activity through disruption of the MIF trimer structure. Ebselen was additionally shown to attenuate glucocorticoid overriding and Akt phosphorylation, but it acted as an agonist of endothelial progenitor cell chemotaxis. This finding not only raises questions about the relevant oligomerization state of MIF-mediated chemotaxis, but by antagonizing certain cellular functions and agonizing others, it also highlights the complexity of MIF signaling and serves as proof of concept that a single small molecule may be capable of silencing certain MIF functions while sparing or promoting others. Another interesting MIF inhibitor identified via HTS is the allosteric inhibitor p425 (**30**, or Pontamine Sky Blue), reported by Bai et al.[122] A member of the azo family of large, sulfonated organic acids, p425 binds the surface of MIF near the interface of two monomers. An X-ray crystal structure showed it forming a cap over the active site and engaging in hydrogen bonds with Lys-32, Asn-109, and Asn-110. In addition to inhibiting tautomerase activity, p425 attenuates CD74 binding (IC_{50} = 0.81 μM), glucocorticoid overriding, secretion of matrix metalloproteinases, and p53-mediated apoptosis. However, it showed little intrinsic affinity for MIF in a fluorescence polarization assay,[109] so the mechanisms for the responses are unclear.

A limitation that high-throughput screening of MIF has heretofore shared with *in silico* screenings is the reliance on inhibition of MIF's tautomerase activity as the primary filtering method for identifying inhibitors. While compounds capable of inhibiting enzymatic activity have been shown to interfere with MIF's biological activities, there is not a strict correlation between the two. Identification of inhibitors like p425 and ebselen with non-canonical modes of inhibition shows that HTS approaches can circumvent the focus on the tautomerase active site. However, both of these inhibitors necessarily inhibited tautomerase activity in order to be identified in the first place. It is possible that a potent allosteric inhibitor of biological activity may be overlooked when employing current HTS methods, if they are not capable of additionally modulating tautomerase activity. Others have proposed that the development of a HTS assay using attenuation of MIF–receptor binding as the metric for activity rather than tautomerase inhibition would be a useful advance for the discovery of MIF inhibitors.[123] In their discovery of p425, Bai et al. employed a CD74 binding assay as a secondary filter of 274 hits from an initial tautomerase HTS of 230,000 compounds. This assay may therefore have the potential to be scaled up to screen large

libraries. Other scalable assays focusing on MIF–chemokine receptor interactions would also prove useful.

2.4 EXISTING DRUGS AND NATURAL PRODUCTS

Clinically approved drugs and natural products comprise the final class of MIF inhibitors. In light of MIF's implication in the pathogenesis of inflammatory and auto-immune disease, a selection of anti-inflammatory drugs and natural products have been purposefully examined for anti-MIF activity or discovered to be MIF inhibitors through screening of whole-cell lysates (Figure 8). While these reports do not necessarily represent a conventional medicinal chemistry approach, the investigation of such compounds presents a way to introduce serendipity to the drug discovery process. These compounds are often already known to possess anti-inflammatory properties, and may exact their activity by interacting with MIF in unprecedented ways, shifting focus from binding to the tautomerase active site to novel modes of MIF inhibition.

In addition to the acetaminophen metabolite NAPQI and ebselen, two other drugs have been found to interact with MIF. Ibudilast (**31**), an anti-inflammatory drug used mainly in Japan for the treatment of bronchial asthma, allergic conjunctivitis, and cerebrovascular disorders,[124] was identified as a MIF inhibitor by Cho et al. in 2010.[125] Ibudilast was found to decrease MIF-mediated chemotaxis and inhibit enzymatic activity (K_i = 30.9 μM) in a noncompetitive manner. X-ray crystal structures of ibudilast bound to MIF showed it binding to an allosteric site adjacent to the tautomerase active site. Ibudilast binding incurs a conformational change in Tyr-36, which alters the dimensions of the tautomerase active site but still permits binding of the substrate HPP. In 2016, Bloom et al. reported that iguratimod (**32**), an anti-inflammatory drug used in China and Japan for the treatment of rheumatoid arthritis, also exhibits anti-MIF activity.[88] Iguratimod inhibits MIF's tautomerase activity (IC_{50} = 6.81 μM), attenuates MIF-mediated release of IL-8 in the absence of LPS, and improves survival in a mouse model of sepsis. The binding mode of this compound, however, was not reported.

A number of natural products have also been shown to inhibit MIF activity, including curcumin, resveratrol, and derivatives thereof;[126–128] a diverse subset of dietary isothiocyanates (including **33**);[129–131] vitamin E;[132] an oxidized form of the dietary flavonoid (−)-epicatechin;[133] and both stereoisomers of the thyroid hormone thyroxine (**34**).[134] Ellagic acid (**35**), a polyphenolic antioxidant, capable of inhibiting enzymatic activity, NF-κB nuclear translocation, and leukocyte chemotaxis was reported to bind to MIF in the cryptic surface binding site previously identified by McLean et al.[104,135]

Two allosteric inhibitors have also been identified. First, epoxyazadiradione (**36**), a limonoid reported by Alam et al. to inhibit MIF's tautomerase activity (IC_{50} = 6.4 μM), induction of iNOS, nuclear translocation of NF-κB, and macrophage chemotaxis, was predicted via molecular docking to bind MIF at one of the poles of the protein's central pore.[136] The inhibitor forms hydrogen bonds with Asn-105 and Asn-102 of one monomer and packs against Tyr-99 of an adjacent monomer. While docking does not always accurately predict the binding modes of MIF inhibitors, the structure of epoxyazadiradione is likely too large to be accommodated in the narrow tautomerase active site, so an allosteric mode of binding

seems reasonable. An epoxyazadiradione–MIF X-ray crystal structure would be useful in validating this novel binding interaction. If this inhibitor does indeed bind to the central pore, its ability to inhibit tautomerase activity is particularly intriguing in that it suggests conformational changes large enough to affect the distal active site. Second, spirohexenolide A (**37**), a polyketide isolated from the soil bacterium *Streptomyces platensis*, was reported by Yu et al. to inhibit MIF-mediated Akt phosphorylation in murine fibroblasts and the uptake of MIF by human colon cancer cells.[114] Interestingly, spirohexenolide A did not inhibit MIF's tautomerase activity, suggesting an allosteric binding mode. Neither molecular docking nor X-ray crystallography experiments have yet been used to elucidate this compound's mode of inhibition, but its size—like epoxyazadiradione—likely precludes binding to the active site.

3. INCONSISTENCIES IN THE LITERATURE

As more MIF inhibitors are discovered, a growing concern is the reproducibility and consistency of the reported results. Difficulty replicating tautomerase assay results is an issue that has been documented with respect to MIF. The IC_{50} of the thoroughly studied MIF inhibitor ISO-1, for example, has been reported as low as 7 μM[87] and as high as >100 μM,[90] with most reports agreeing on a value closer to 18 μM.[84,88,113] A study conducted by our laboratory using the HPP tautomerase assay highlighted inconsistencies in the literature by screening a subset of reported MIF inhibitors side-by-side.[102] Minor, systematic shifts in values might be expected when assays are performed in different laboratories, but the results of our study showed much greater variations, with some inhibitors exhibiting activities orders of magnitude different from their original values. There are a few possible explanations for these inconsistencies.

The tautomerase substrate may be a contributing factor. There are two commonly used protocols for assessing tautomerase inhibition: the dopachrome and HPP assays. The dopachrome assay, which can utilize D-dopachrome or the D/L-dopachrome methyl ester as substrate, is the more widely used protocol, but it suffers from a few drawbacks. Firstly, the dopachrome substrates are photosensitive and are stable only over a short timeframe (1–2 hours when stored at 4 °C).[120] Secondly, the linear range for the kinetic assay is short (ca. 40 seconds). The potential introduction of error through substrate decomposition or inaccurate initial velocity measurements have led some groups to argue for use of HPP instead, which is stable on the order of days and has a longer linear range in the kinetic assay (2–3 minutes).[102,120] In addition to substrate-related error, protein-derived error is another possible source of inconsistency, with protein integrity being a primary concern. The potential for fluctuations in activity arising from the use of disparate batches of protein has led to the recommendation that future publications properly identify the source and purification methods of recombinant MIF used in assays.[108,123] Additionally, the assaying of well-studied inhibitors, like ISO-1 and Orita-13, as controls alongside new MIF inhibitors is essential each time the assays are run to validate the accuracy of the results and contextualize them within the larger MIF inhibitor literature.

At this point, combining results from different tautomerase assays to develop SAR models or train scoring functions for docking is ill-advised. Too many inconsistencies arise from the

combination of non-covalent, covalent, and allosteric inhibition, in addition to the dependence of IC_{50} values on the choice of substrate and its concentration. It would be helpful if researchers in the area uniformly reported K_i results instead, since they remove the latter variables.

Concerns have also been raised over the conditions under which some of the cellular assays have been conducted. Just as poorly characterized protein can frustrate the accuracy of enzyme assays, impure MIF will be of little use in probing the efficacy of potential therapeutics. The use of fusion agents as affinity tags, for example, can affect biological activity, and even though the purification of MIF is straightforward enough that this practice is often unnecessary, general reporting of affinity tagging has been poor despite its biological implications.[123,137] Bacterial expression of recombinant MIF also introduces the possibility of contamination with LPS, which introduces its own biological activity.[123] LPS contamination is of particular concern in assays that use the expression or secretion of other cytokines as the metric for activity (e.g., glucocorticoid overriding assays). Kudrin et al. have shown that highly pure, LPS-free MIF was capable of releasing only IL-8 and had no effect on the release of other cytokines.[36] MIF did, however, exhibit pronounced synergistic effects with LPS on TNF-α and IL-β1 release and retained its ability to override glucocorticoid-mediated inhibition of TNF-α, IL-6, and IL-β1 production. These findings raise questions about some activities attributed to MIF (e.g., TNF-α secretion) and subsequent reports of inhibitors capable of ameliorating those activities. Bloom et al. have also shown that some MIF inhibitors, including ISO-1 have what appear to be promiscuous anti-inflammatory activity in that they can inhibit TNF-α release from LPS-treated monocytes in the absence of exogenous MIF.[88] This finding might imply that these inhibitors are interacting with other proteins involved in the immune response, though an alternative explanation is that they may be effecting their activity by permeating the cell and modulating endogenous, intracellular MIF. Together, these reports illustrate the importance of thoroughly characterizing recombinant MIF protein, reporting expression conditions and endotoxin content of purified products, and performing well-designed control experiments to validate that inhibitor effects are MIF-specific.

4. MODES OF INHIBITION: A CLOSER LOOK

MIF, with its numerous binding partners and upstream role in inflammatory and proliferative signaling, is a potentially attractive target for drug development. Though inhibition of protein–protein interactions is a challenging endeavor, the scientific literature abounds with diverse small molecule binders of MIF capable of disrupting its many functions. The greatest boon to the discovery of MIF inhibitors is the presence of the tautomerase active site, which has been coopted as the binding site for the vast majority of MIF inhibitors, with both covalent and non-covalent binding modes possible.

Covalent modification of the active site requires nucleophilic attack of Pro-1. A number of common, covalent-binding motifs have shown efficacy, including Michael acceptors,[138] isothiocyanates,[131] benzisothiazolones,[98] and carbonyl compounds capable of nucleophilic acyl substitution.[100] Minor structural changes of a covalent binder capable of repositioning the inhibitor in the active site may cause a shift to non-covalent binding. Demethylation of

the covalent benzisothiazolone inhibitor **8**, for example, is expected to move the inhibitor deeper inside the binding pocket, relocating the electrophilic moiety further away from Pro-1 to allow for reversible inhibition (compound **9**).[102] Misidentification of covalent MIF inhibitors as reversible inhibitors is not an uncommon occurrence. It is important to scrutinize the proximity of electrophilic ligand sites to the highly nucleophilic Pro-1 and to perform experiments that validate binding mode. The size and shape of the active site is also an important factor to consider when determining the binding mode of novel inhibitors. That the active pocket is fairly narrow generally precludes binding of non-linear compounds. A few pyramidal inhibitors (including **13** and **28**) that would have difficulty fitting in the pocket, for example, were originally reported as non-covalent[96,113,121] and later found to form adducts with Pro-1 through displacement of morpholine or anilinyl rings.[102,112] A final note on non-reversible inhibition of MIF is that while covalent modification of Pro-1 presents a therapeutic strategy, there is no significance in the reported IC_{50} and K_i values, as they are variable depending on the incubation time of the protein with the inhibitors.[102]

Reversible binders of the tautomerase active site are the most common class of MIF inhibitors. Most of these inhibitors form hydrogen bonds with Pro-1, Lys-32, Ile-64, and/or Asn-97 and engage in aryl–aryl interactions with Tyr-36, Tyr-95, and/or Phe-113. Compounds that hydrogen bond with Asn-97 at the back of the binding pocket often do so through a phenol ring.[83,87,107] SAR studies have shown that the pocket can accommodate *ortho*-fluorination of this ring, which results in a boost in affinity.[93,108] Our lab has recently shown that a pyrazole ring can serve as an alternative to the phenol that may overcome some of the metabolic concerns associated with phenols.[110] Lys-32, which is located near the rim of the active site, has been shown in crystal structures to form multiple hydrogen bonds with ligands, though it was recently found that there may be a limit to the benefits of increased coordination number of the ammonium side chain.[139] Specifically, the naphthyridinone **21** (Figure 6) was found to have similar K_i and K_d values as related quinolines such as **20** in spite of an added hydrogen bond from Lys-32 to the carbonyl oxygen atom, as seen in crystal structures.[139] Crystallography and modeling also made it clear that substituents at the 6- and 7-positions of the quinolines, e.g., **19**, should protrude from the binding site and be solvent-exposed. This feature has been used to advantage to modulate aqueous solubility for this series from ca. 2 to 1000 μg/mL.[140] It can also be used to modulate the contact between inhibited-MIF and receptors such as CD74.

Outside of the binding pocket, MIF has three conserved cysteine residues with known relevance to protein function.[27,30,49] Though only one inhibitor has thus far been reported to covalently modify any of these residues, targeting MIF's cysteines is an unexplored strategy that may prove promising. By forming an adduct with Cys-80, ebselen destabilizes the MIF trimer.[121] This mode of inhibition has therapeutic potential, as compounds that effect their activity through trimer disruption may completely abolish trimer-associated activity while sparing functions attributed to MIF monomer (e.g., molecular chaperone activity).[8,58] Cys-80 may be appealing as a covalent binding target for other reasons. Firstly, Cys-80 has been shown to be essential in stabilizing the p53–Mdm2 complex, with a C80S point mutation failing to recapitulate the anti- apoptotic effects of wild-type MIF.[56] Targeting this residue may therefore be a productive strategy for reversing MIF's proliferative effects.

Secondly, Cys-80 is believed to act as a "switch cysteine" for the conversion of redMIF to the disease-related isoform oxMIF.[30] Covalent modification of Cys-80 with a series of pro-oxidative reagents showed that while some precipitated the conversion to oxMIF, at least one covalent adduct remained in the reduced form. This finding might indicate that a covalent inhibitor may be capable of trapping the comparatively benign redMIF oxidation state. Two other cysteine residues, Cys-56 and the less accessible Cys-59 are present in the protein and are contained within the CALC motif responsible for TPOR activity. While the desmethoxy analog of **8** shows some attachment in this region, deliberate targeting of these residues is desirable for a few reasons. Firstly, inhibitors targeting the CALC motif may be useful probes in understanding what, if any, role MIF's TPOR activity plays in cells. Secondly, some of MIF's binding partners are expected to bind in this region. A C59S MIF mutant was unable to replicate MIF effects on JAB1 activity, and a 16-residue MIF peptide fragment spanning the CALC domain strongly competed with the full protein for JAB1 binding.[49] Peptide array analysis and molecular dynamics simulations have suggested that MIF binding to CXCR2 follows the canonical 2-site binding mechanism of classical chemokines.[119,141] MIF's N-loop (an important binding motif for chemokine–chemokine receptor interactions) for CXCR2 binding is predicted to be in the region of the CALC domain while its pseudo-(E)LR motif (another important domain for chemokine–chemokine receptor interactions) is on a proximal loop in the folded protein. Covalent modification of Cys-56 or Cys-59 may therefore inhibit downstream effects of MIF–JAB1 or MIF–CXCR2 interactions while sparing the signaling of other receptors. Finally, this region is expected to undergo a significant conformational shift while converting from redMIF to oxMIF, and a well-designed cysteine-binding inhibitor may be capable of trapping the protein as the former.

In addition to ebselen, four more allosteric inhibitors of MIF have been identified: ibudilast[125] and p425[122] bind near the active site, but epoxyazadiradione[136] and spirohexenolide A[114] are expected to bind distally (Figure 9). Epoxyazadiradione is predicted to bind to MIF's central, solvent-filled pore, but the binding site of spirohexenolide A has not been determined. X-ray crystal structures of these two inhibitors bound to MIF would be valuable in validating or revealing novel binding sites for future MIF inhibitor design. Because of MIF's pleiotropic nature, allosteric inhibitors may be of particular interest, as they have the potential to bind to regions important for the recognition of specific receptors while preserving the integrity of other receptor binding sites. As more becomes known of the motifs and regions essential for MIF's many interactions, allosteric modulation of MIF activity may become a more precise and desirable method for designing targeted therapeutics.

5. PERSPECTIVES

With all but five reported MIF inhibitors eliciting activity by binding to the tautomerase active site, the question at hand is: how reliable is this strategy for developing MIF-directed therapeutics? Tautomerase inhibition is the most commonly reported metric of potency for new MIF inhibitors, and it remains the most convenient way to determine affinity and specificity for MIF *in vitro*; however, both mutational studies and inhibitor screenings have shown that loss of enzyme activity does not necessarily correlate with loss of biological activity.[24–26] Some potent inhibitors of tautomerase function have been found to have no

affect on biological activity[88,115] while others exhibit far-ranging inhibition of cellular function.[89,103,121] Of the receptors MIF is known to engage with, CXCR4 and CD74 are expected to interact with residues near the active site. MIF's N-loop for CXCR4 binding (residues 67–81) and the recently identified tripeptide Arg-86-Leu-87-Arg-88 are particularly important for interactions with the chemokine receptor but are distal to the tautomerase active site.[118,142] Even so, both N-terminal mutation and incubation with ISO-1 have been shown to specifically inhibit MIF–CXCR4 binding.[118] Regarding CD74, mutational studies and computational modeling have identified residues in the proximity of MIF's active site—but not necessarily Pro-1—as relevant for receptor recognition.[116,117] Pantouris et al. showed that inhibitors binding to the active site could reliably inhibit CD74 binding if they extended out of the pocket to penetrate the protein–solvent interface.[116] Though this finding may indicate that merely binding the active site may not be enough to inhibit receptor interactions, other studies have shown that certain active site inhibitors are capable of eliciting far-ranging conformational changes in the protein that can disrupt the recognition motifs of MIF receptors.[143,144] While purposeful design of such compounds may be difficult, since induced conformational changes are challenging to predict, it seems clear that targeting the active site remains a convenient and efficacious strategy for developing MIF inhibitors.

As the pleiotropic functions of MIF continue to be elucidated, it has become common for new inhibitors to be screened with a subset of the growing number of biological assays available. While the tautomerization assay has always been standard in the reporting of new MIF inhibitors, no biological assay has emerged from the morass as equally requisite. As a result, we might learn of a novel inhibitor's ability to reduce nuclear translocation of NF-κB or attenuate Akt phosphorylation or inhibit expression of one of many proinflammatory factors, but given the complex and interconnected nature of MIF signaling, such discrete downstream effects are not always constructive for directly comparing the efficacy and modes of action of different inhibitors. While it would be unreasonable to expect an exhaustive screening of new inhibitors against the full spectrum of MIF activities, an arbitrary selection of biological assays makes side-by-side comparisons of inhibitors difficult. Instead, future inhibitor screenings might focus on assays that measure cellular responses that are directly responsible for disease phenotypes, such as cytokine production and secretion, cell proliferation and apoptosis, or chemotaxis. Another approach may be the employment of assays that are dependent on specific, well-characterized receptor interactions to deduce which crucial protein–protein interactions a new inhibitor is disrupting rather than assays that measure downstream effects which might be triggered by multiple different cascades or whose upstream regulators are unknown. A benefit of prioritizing assays that measure the inhibition of specific MIF–receptor interactions is the possible identification of inhibitors capable of attenuating certain interactions while sparing others. While a pharmaceutical capable of inhibiting the majority if not the sum total of MIF's biological activities may be desirable in some disease models, selective modulation of particular MIF-related activities could be advantageous in the treatment of certain conditions, including ischemia,[107] multiple sclerosis,[88] and ALS.[58]

The field of MIF inhibition may be at the cusp of great advancement. A large number of diverse MIF inhibitors have been reported, and the extensive structure–activity relationship

data of well-developed series like the isoxazoles, triazoles, and pyrazoles will be invaluable in progressing the efficacy of other promising scaffolds. Looking forward, potentially groundbreaking discoveries may arise from probing the features of active-site inhibitors that induce widespread conformational changes, targeting MIF's cysteine residues as a means to address redox-related functions and oxMIF pathology, or the development of new classes of allosteric inhibitors that target specific MIF–receptor interactions. The development of new biological assays, preferably ones that can be scaled up for high-throughput screening, will be indispensable in making great strides in the field. Currently, oxMIF-specific antibodies are in clinical trials, and while a non-biological inhibitor has yet to progress to this stage, it seems highly likely that a small molecule MIF-directed therapeutic is within reach.

ACKNOWLEDGMENT

Gratitude is expressed to the U. S. National Institutes of Health (GM32136) for research support.

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ABBREVIATIONS USED

MIF	macrophage migration inhibitory factor
HPP	4-hydroxyphenylpyruvate
TPOR	thiol-protein oxidoreductase
redMIF	reduced MIF
oxMIF	oxidized MIF
LPS	lipopolysaccharide
TNF-α	tumor necrosis factor

MAP	kinase mitogen activated protein kinase
ERK1/2	extracellular signal-regulated kinases
JAB1	jun-activation domain-binding protein 1
TXNIP	thioredoxin-interacting protein
HTS	high-throughput screening

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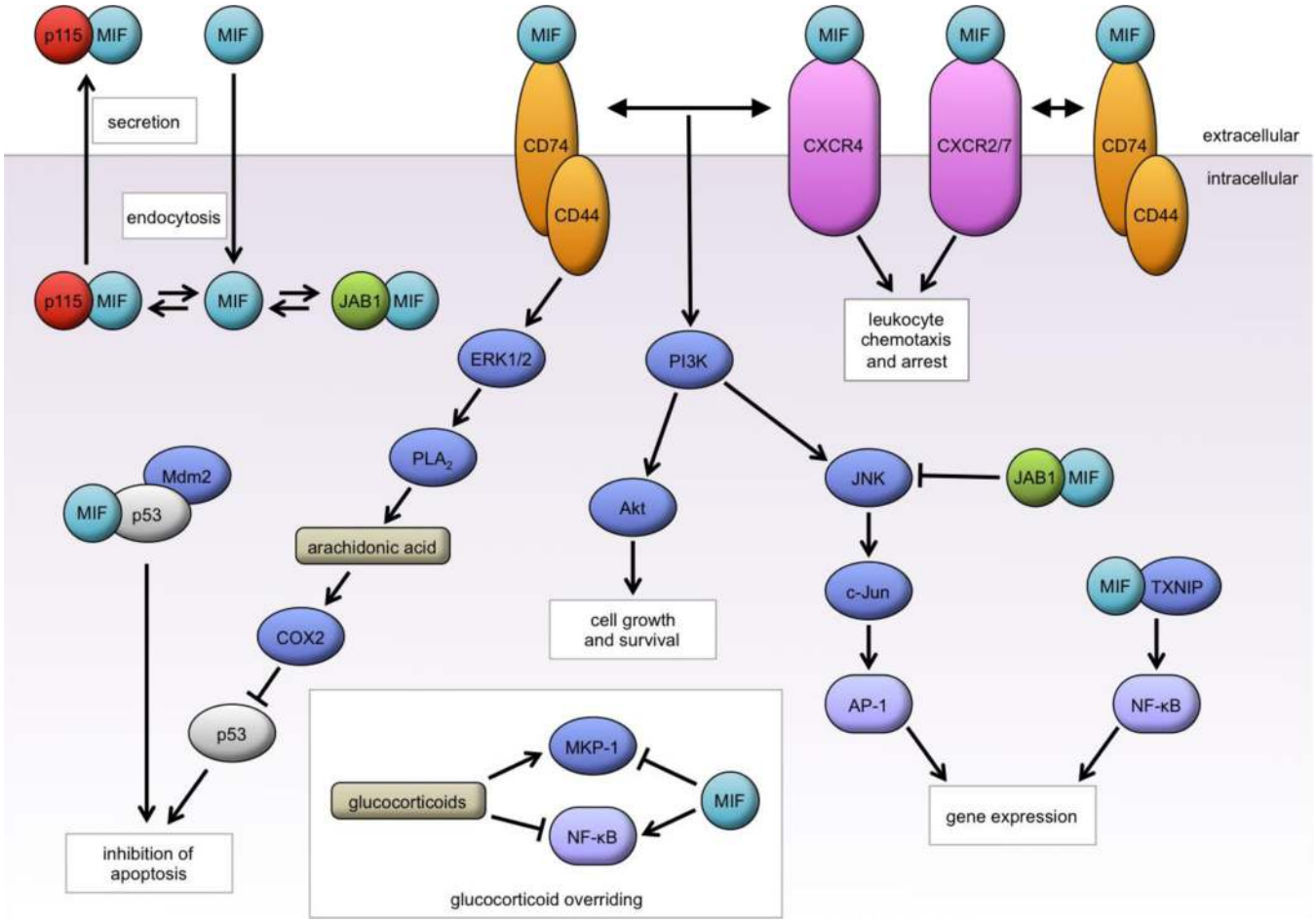


Figure 1.
Signal transduction pathways regulated by MIF.

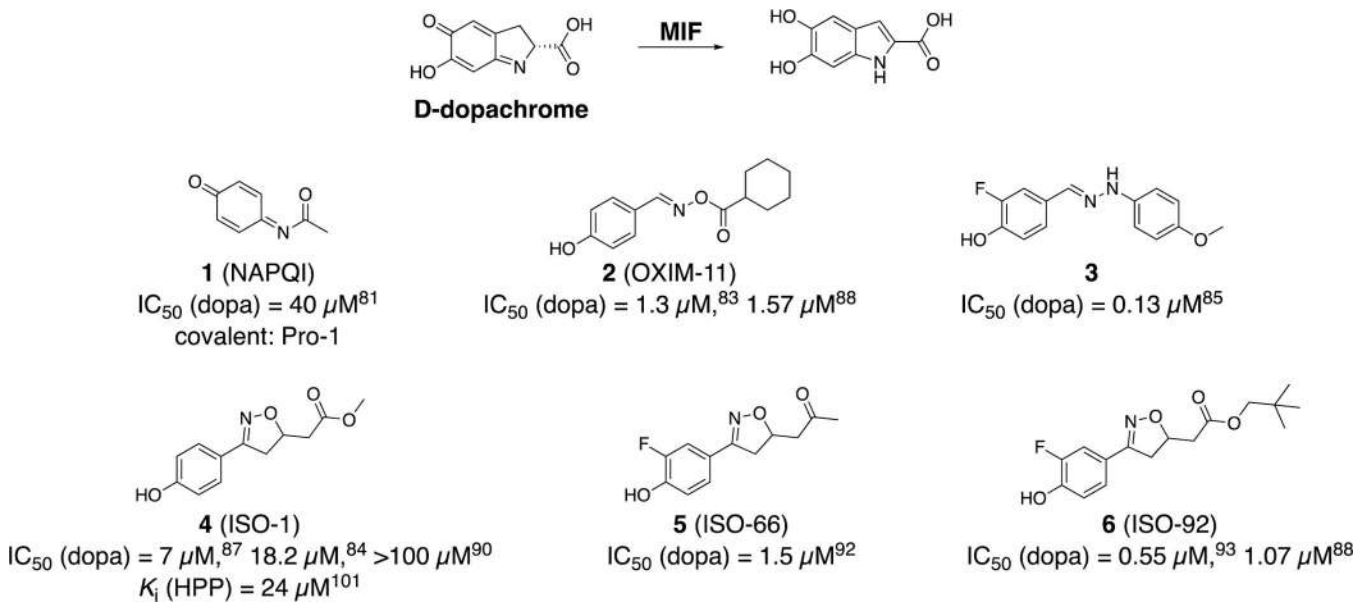


Figure 2.

A selection of dopachrome analog MIF inhibitors. Relevant references are noted as superscripts.

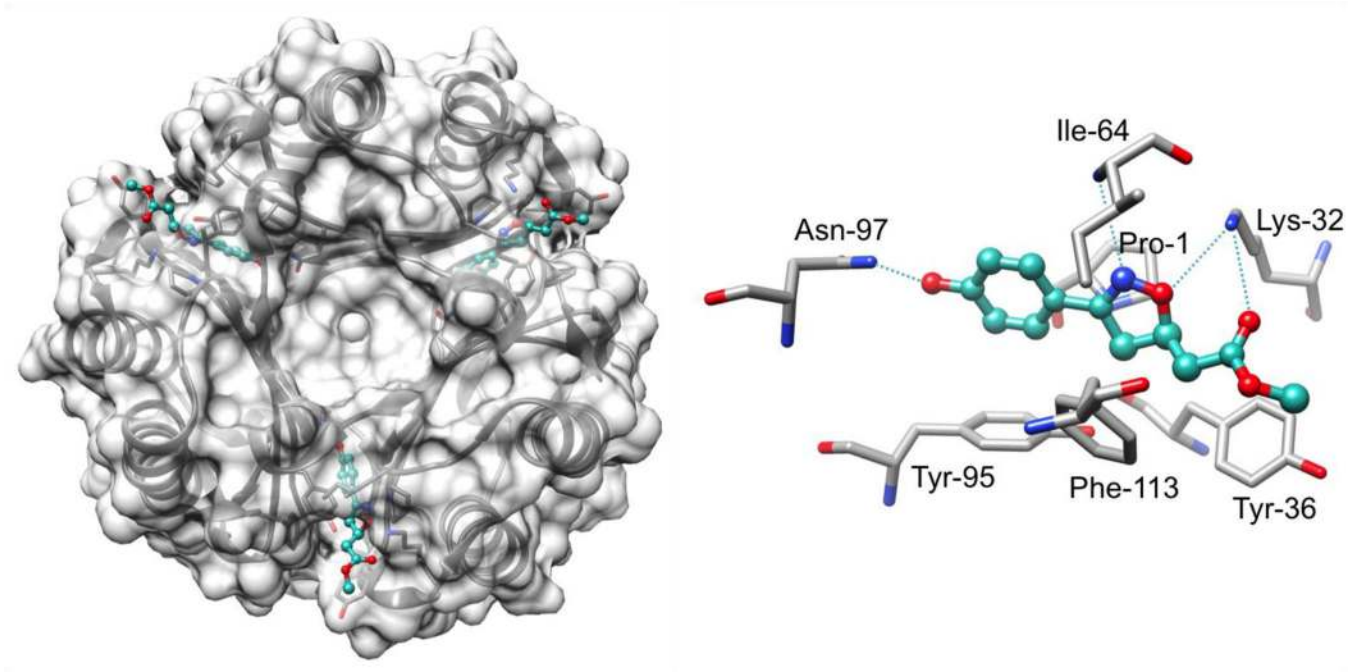


Figure 3.

X-ray crystal structure of ISO-1 (cyan) bound to MIF. The inhibitor occupies all three active sites (left) and forms hydrogen bonds with the active site residues Lys-32, Ile-64, and Asn-97 (right). PDB ID: 1LJT.[87]

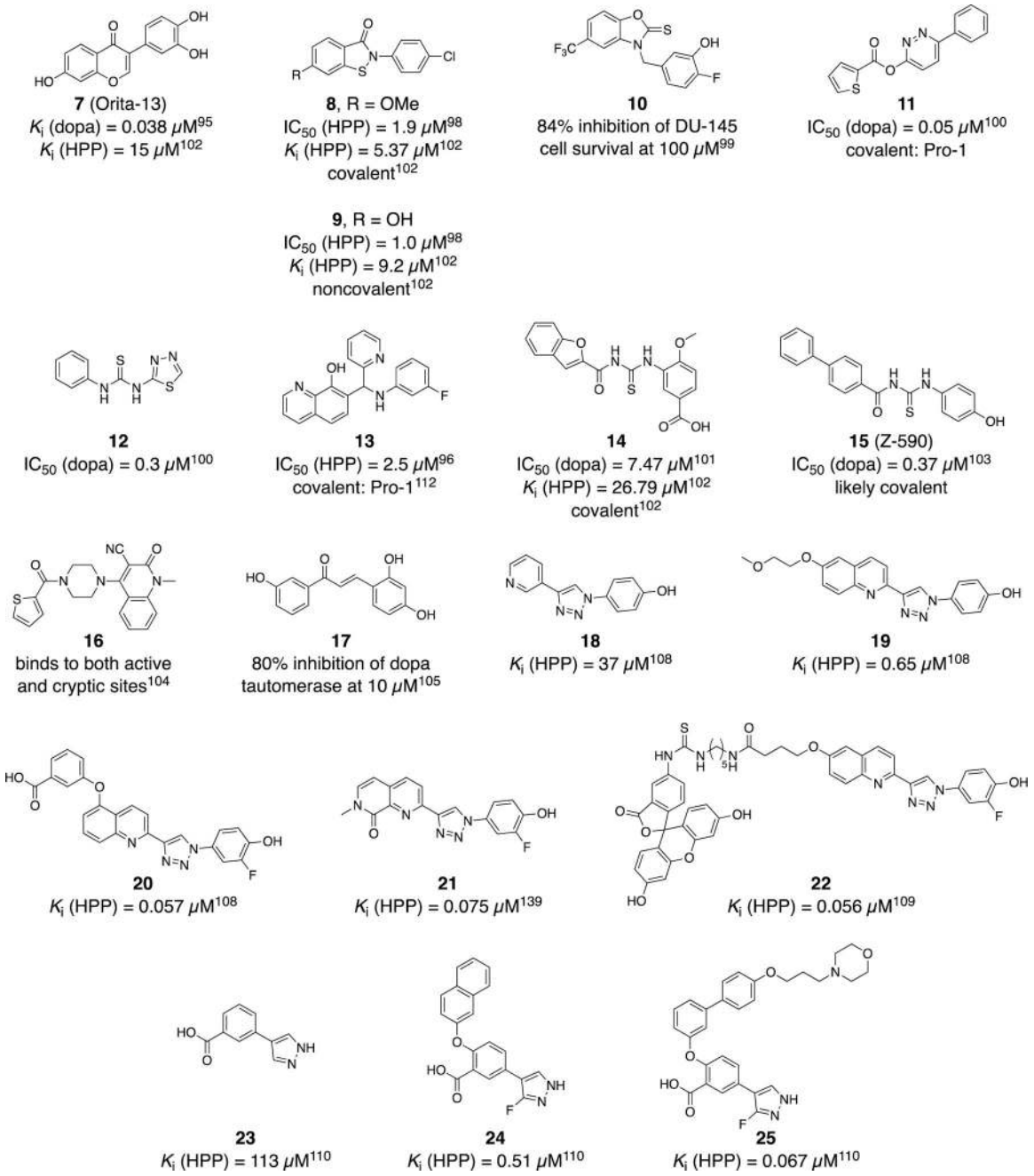


Figure 4.

A selection of MIF inhibitors identified or developed with the aid of *in silico* methods.

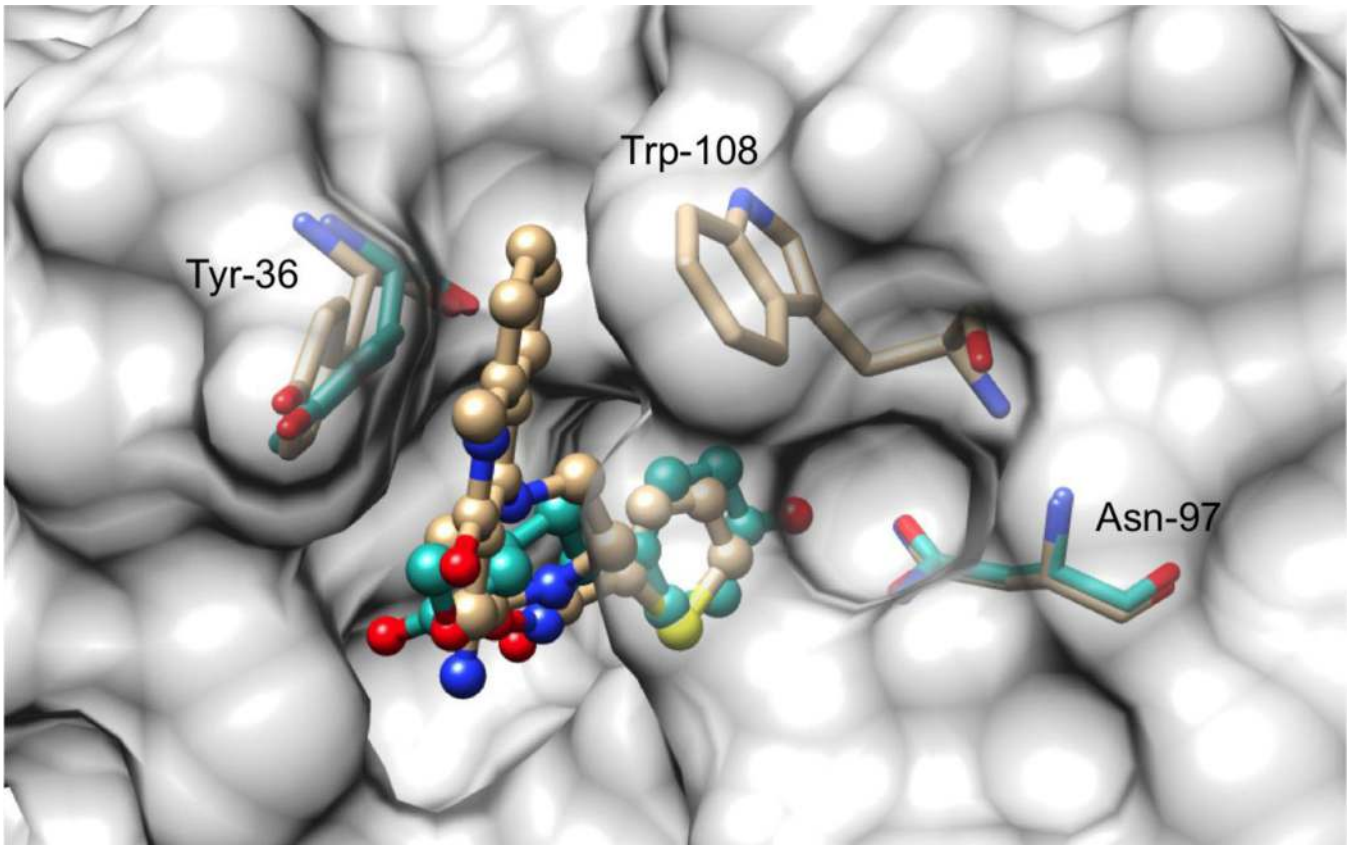


Figure 5.
Overlay of X-ray crystal structures of ISO-1 (cyan) and **16** (tan) showing occupation of both the active site and the cryptic site by the latter. PDB IDs: 1LJT,[87] 3L5T.[104]

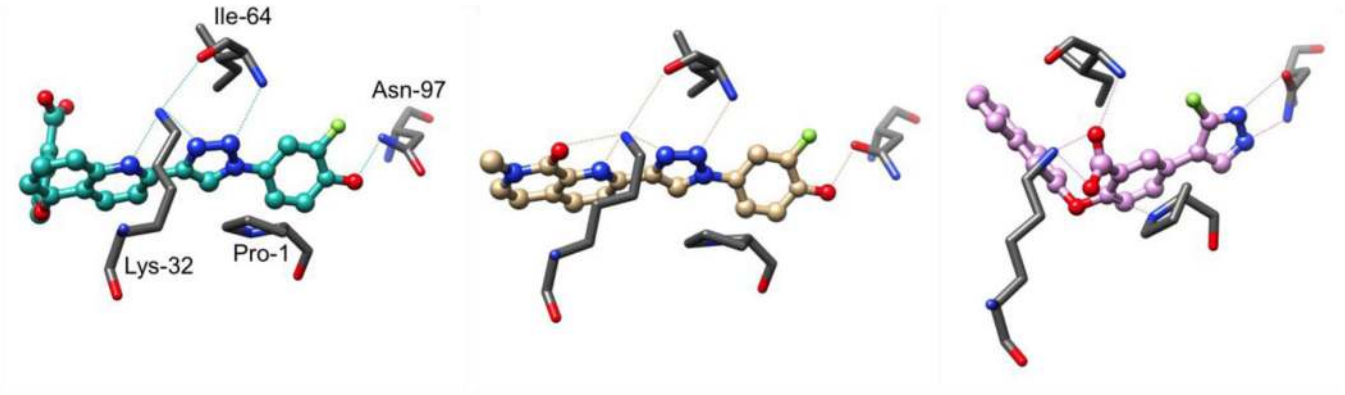
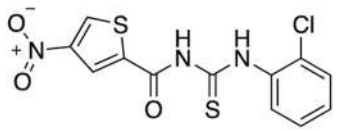
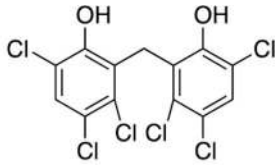


Figure 6.

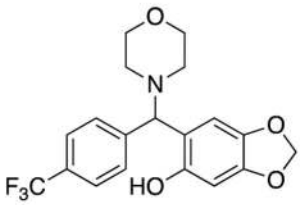
X-ray crystal structures of **20** (cyan), **21** (tan), and **24** (pink), showing key interactions with active site residues. PDB IDs: 5HVS,[109] 6B1C,[139] 6CBH.[110]



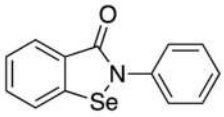
26
IC_{50} (dopa) = 7.08 μM[121]
K_i (HPP) = 2.47 μM[121]
covalent: Pro-1



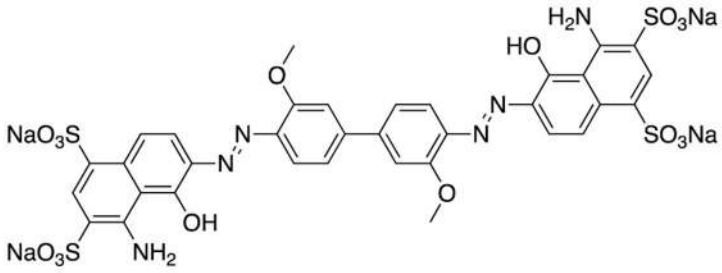
27
IC_{50} (dopa) = 6.00 μM[121]
K_i (HPP) = 5.55 μM[121]



28
IC_{50} (dopa) = 2.40 μM[121], 6.12 μM[113]
IC_{50} (HPP) = 6.44 μM[113]
K_i (HPP) = 0.57 μM[121], 0.052 μM[102]
covalent: Pro-1[102]



29 (ebselen)
IC_{50} (dopa) = 2.40 μM[121]
K_i (HPP) = 0.57 μM[121]
covalent: Cys-80
trimer disrupter



30 (p425)
>50% HPP tautomerase inhibition at 12 μg/mL[122]
IC_{50} (CD74 binding) = 0.81 μM[122]
allosteric binder: protein surface

Figure 7.
A selection of MIF inhibitors identified via HTS.

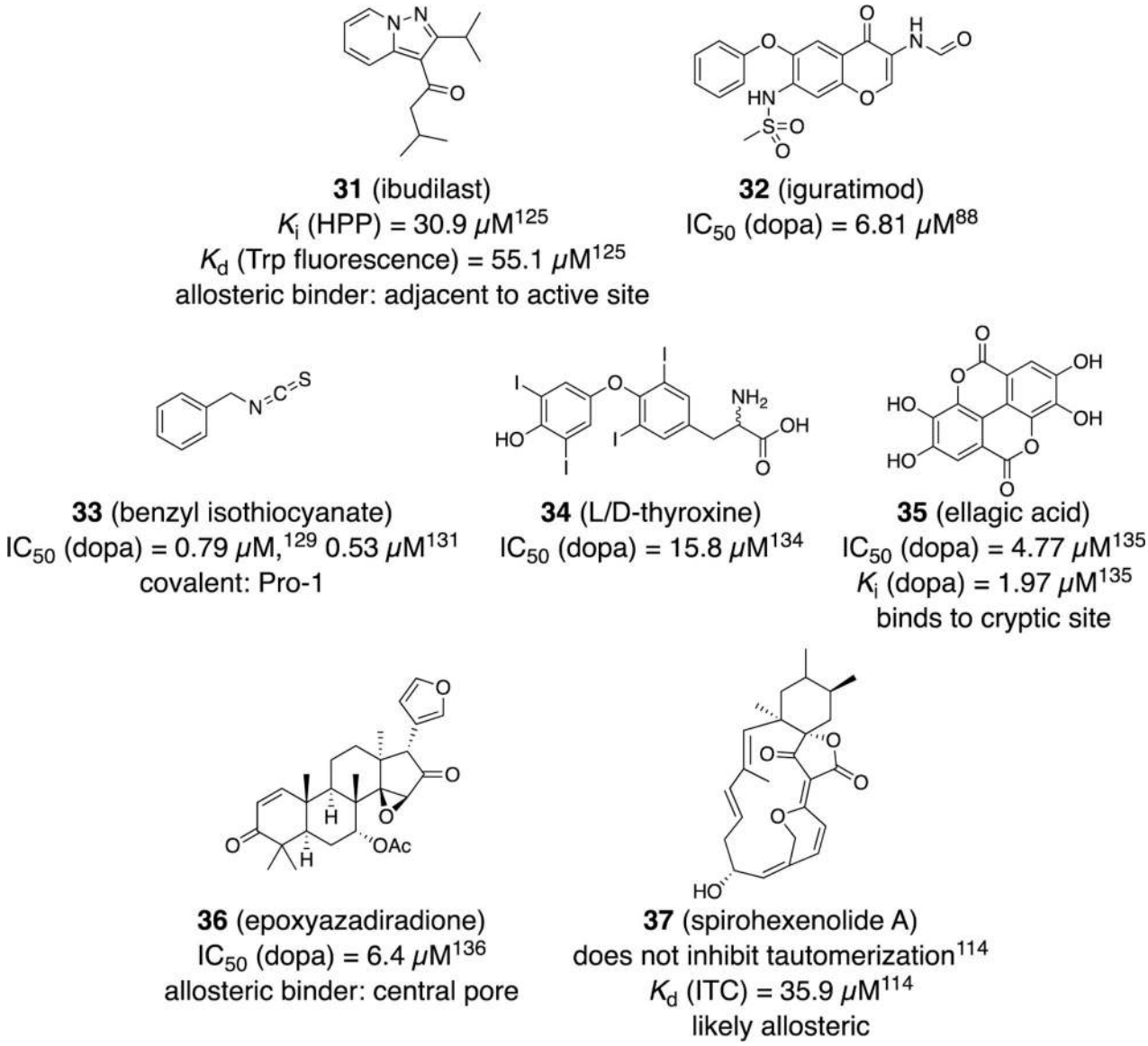


Figure 8.
A selection of approved drug or natural product MIF inhibitors.

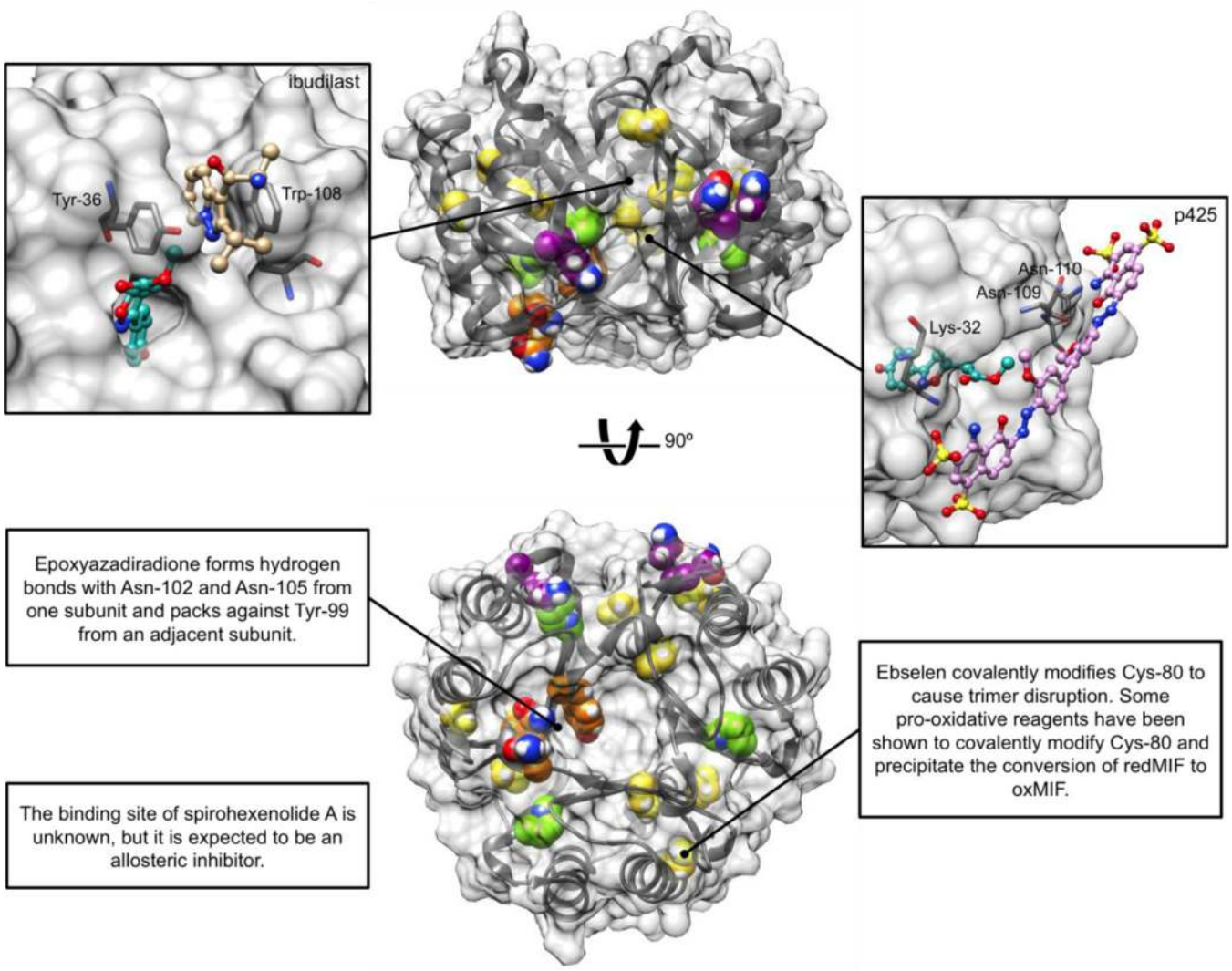


Figure 9.

Allosteric inhibitors of MIF and their binding sites. In the central images, Pro-1 (green), MIF's three cysteine residues (yellow), and key residues of the p425 (purple) and epoxyazadiradione (orange) binding sites are shown. Overlays of the X-ray crystal structure of ISO-1 (cyan) with crystal structures of ibudalist (tan, left) and p425 (pink, right) are also shown. PDB IDs: 6B1K,[139] 1LJT,[87] 3IJG,[125] 3U18.[122]